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The antiproliferative function of violacein-like purple violet pigment (PVP) from an Antarctic *Janthinobacterium* sp. Ant5-2 in UV-induced 2237 fibrosarcoma

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Abstract

Background—In this study, we have investigated the chemotherapeutic potential of a purple violet pigment (PVP), which was isolated from a previously undescribed Antarctic *Janthinobacterium* sp. (Ant5-2), against murine UV-induced 2237 fibrosarcoma and B16F10 melanoma cells.

Methods—The 2237, B16F10, C50, and NIH3T3 cells were treated with PVP at different doses and for different times, and their proliferation and viability were detected by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Cell cycle arrest induced by PVP in 2237 fibrosarcoma cells was assessed by flow cytometry and expression analysis of cell cycle regulatory proteins were done by Western blot. Apoptosis induced by PVP in 2237 cells was observed by annexin-V/propidium iodide double staining flow cytometry assay and fluorescence microscopy. To further determine the molecular mechanism of apoptosis induced by PVP, the changes in expression of Bcl-2, Bax and cytochrome c were detected by Western blot. The loss of mitochondrial membrane potential in PVP treated 2237 cells was assessed by staining with JC-1 dye following flow cytometry. Caspase-3, Caspase-9 and PARP cleavage were analyzed by Western blot and Caspase-3 and -9 activities were measured by colorimetric assays.

Results—*In vitro* treatment of murine 2237 cells with the PVP resulted in decreased cell viability (13–79%) in a time (24–72 h) and dose (0.1–1 μM)-dependent manner. The PVP-induced growth inhibition in 2237 cells was associated with both G0/G1 and G2/M phase arrest accompanied with decrease in the expression of cyclin dependent kinases (Cdks) and simultaneous increase in the expression of cyclin dependent kinase inhibitors (Cdki) – Cip1/p21 and Kip1/p27. Further, we observed a significant increase in the apoptosis of the 2237 fibrosarcoma cells which was associated with an increased expression of pro-apoptotic protein Bax, decreased expression of anti-apoptotic proteins Bcl-2, disruption of mitochondrial membrane potential, cytochrome c release, activation of caspase-3, caspase-9 and poly-ADP-ribose-polymerase (PARP) cleavage.

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Conclusions—We describe the anti-cancer mechanism of the PVP for the first time from an Antarctic bacterium and suggest that the PVP could be used as a potent chemotherapeutic agent against nonmelanoma skin cancers.

Introduction

Skin cancer is a growing health problem around the globe. The ultraviolet B (UVB) (290–320 nm) component of the solar radiation causes cumulative damage of the skin cells resulting in immunosuppression that leads to skin cancer.[1] It has been reported that the exposure to UV radiation increases risk of both the melanoma and non-melanoma skin cancers in humans.[2] Moreover, it has been estimated that approximately one million new cases of nonmelanoma skin cancers were diagnosed in 2008 in USA alone causing nearly 1000 deaths (National Cancer Institute, http://www.cancer.gov/). Recently, there has been a considerable effort in the development of chemopreventive as well as chemotherapeutic agents from naturally occurring plant sources such as green tea,[3] resveratrol[4], and grape seed proanthocyanidins[5] for the prevention and/or treatment of skin cancers. However, little attention has been given to the identification and isolation of natural compounds from microorganisms such as the chicamycin[6] and violacein[7] that possess the anti-tumor activity. Although violacein, which has been isolated from *Chromobacterium violaceum,* has gained some attention because of its antitumoral, antibacterial, antiulcerogenic, antileishmanial, and antiviral activities,[8,9] still little work has been done to elucidate the mechanism of action of this compound on diseased cells.

Violacein from *C. violaceum* is characterized as 2-dihydro-5-(5-hydroxy-1H-indol-3-yl)-2-oxo-3H-pyrrol-3-ilydene)-1, 3-dihydro-2H-indol-2-one, formed by the condensation of two modified L-tryptophan molecules.[8] The cytotoxic function of violacein has been reported in HL60 cells through the activation of caspase-8, transcription of nuclear factor κB (NF-κB) target genes, and p38 mitogen-activated protein (MAP) kinase.[10] It is thought that violacein resembles tumor necrosis factor α (TNF-α) in action by activating TNF receptor 1.[10] Further, violacein mediated ROS production is thought to activate caspase-3, release of cytochrome c, and calcium release to cytosol in Caco-2 cells.[11] However, the cytotoxic function of the *C. violaceum* violacein has not been tested on skin cancer cell lines. In our study, a violacein-like purple violet pigment (PVP) was extracted and purified from an Antarctic bacterium, and for the first time we attempted to examine the chemotherapeutic effect of this pigment on skin cancer cells *in vitro*. Our study also provides insights into the mechanism by which this compound inhibits cell cycle progression and induces apoptosis in skin cancer cells via modulations in the mitochondrial pathway and the Bcl-2 family of proteins.

Materials and Methods

Cell lines, bacteria, and reagents

Murine UV-induced 2237 fibrosarcoma and C50 normal keratinocyte cell lines were a kind gift from Dr. Ananthaswamy and Dr. Susan Fisher (Houston, TX, USA) respectively, to Dr. Nabiha Yusuf. Murine fibroblast NIH3T3 and B16F10 melanoma cell lines were generously provided by Dr. Rui-Ming Liu and Dr. Zeng Bian-Zhu (UAB) respectively. *Janthinobacterium* sp. (Ant5-2) was isolated from a Proglacial Lake P9 (also known as Lake Podprudnoye) located in the Schirmacher Oasis, Dronning Maud Land of East Antarctica. All primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); except cyclinB1 and cytochrome c antibodies that were from eBioscience (San Diego, CA, USA); Cdc2 from BioLegend (San Diego, CA, USA), β-actin from Bethyl Laboratories Inc. (Montgomery, TX, USA) and anti–poly-ADP-ribose-polymerase from Upstate Cell Signaling Solutions (Lake Placid, NY, USA). The Vybrant apoptosis assay kit #2 was

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purchased from Molecular Probes Inc. Invitrogen (Carlsbad, CA, USA). The fluorescent dye JC-1, caspase-9 substrate and caspase-3 substrate (chromogenic) were purchased from AnaSpec and A.G. Scientific, Inc. (San Diego, CA, USA), respectively.

Purification and characterization of PVP

The PVP from Ant5-2 was first partially purified by liquid chromatograph reverse phase flash column [C18 stationary phase; carbon 23%; particle size 40–60 μm, methanol/water (75 : 25) as mobile phase]. This partially purified pigment was further purified by reverse phase HPLC column. Additionally, PVP was analyzed by Mass spectra using Micromass Electrospray Ionization Mass Spectrometer and an HP 1100 LC Micromass Platform LCZ with a C18 Column and proton (^{1}H) NMR (Bruker ARX 700 spectrometer; Bruker Corporation, Madison, WI, USA). The concentration of PVP was determined by spectrophotometer.[7] The chemical structure of PVP was drawn using BKchem software (http://bkchem.zirael.org/index.html).

Cell culture conditions

All the cell lines were cultured as monolayer in Dulbecco's Modified Eagle's Medium supplemented with 10% heat inactivated fetal bovine serum (Hyclone, Logan, UT, USA), 100 μg/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA), and maintained in a humidified atmosphere of 95% air and 5% CO2 at 37 °C.

MTT assay for cellular viability

MTT assay is a colorimetric assay for measuring the activity of the cellular reductase enzymes in viable cells that reduce yellow 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St Louis, MO, USA) substrate to purple formazan crystals. Briefly, 5×10^4 cells/well in 1 ml of complete culture medium was plated in 24-well culture plates. After overnight incubation, the cells were treated with DMSO as a control, or 0.1, 0.2, 0.5, and 1 μM PVP dissolved in DMSO (0.02% v/v) and further incubated for a 24, 48, and 72 hours at 37 °C in a humidified chamber. At the end of the stipulated period, MTT (250 μl of 50 μg/ml stock) was added into each well and incubated for 2 hours. The resulting formazan was then dissolved in 500 μl of DMSO and absorbance of the formazan in each well was recorded at 540 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell cycle progression analysis by flow cytometry

The effect of PVP treatment on distribution of cells in different phases of the cell cycle was analyzed by flow cytometry. Briefly, 2237 fibrosarcoma cells were grown in complete culture medium for 24 h. After overnight serum starvation, the cells were treated with varying concentrations of PVP (0, 0.1, 0.2, 0.5, and 1.0 μM). After 24 hours, 1×10^6 cells were collected and resuspended in 50 μl of cold (4 °C) PBS (pH 7.4), cold methanol (450 μl) was added and incubated at 4 °C for 1 hour. The cells were centrifuged at 1400 *g* for 5 minutes at 4 °C, washed with cold PBS (pH 7.4); resuspended in 500 μl PBS; and incubated with 5 μl RNase (20 μg/ml final concentration) (Ambion, Inc., Austin, TX, USA) for 30 minutes and propidium iodide (50 μg/ml final concentration) for 1 hour in the dark. The cell cycle distribution of the cells was then determined using a BD FACSCalibur™ Flow Cytometer (BD Biosciences, San Jose, CA, USA) equipped with BD FACS Diva software. ModFit LT 3.0 cell cycle analysis software was used to determine the percentage of cells in the different phases of the cell cycle.

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Apoptosis assessment by annexin-V/propidium iodide (PI) staining

For the detection of apoptotic and necrotic cells, Vybrant Apoptosis Assay Kit #2 (Molecular Probes Inc., Eugene, OR, USA) was used according to the manufacturer's protocol. After 48 hours treatment with PVP, the cells were resuspended in 1× annexin binding buffer and incubated with Annexin V Alexa 488 and PI for cellular staining in dark and cells were either analyzed by FACS using a BD FACSCalibur™ Flow Cytometer equipped with BD FACS Diva software or an Olympus IX70 (Thornwood, NY, USA) fluorescence microscope. Confocal images of green annexin-Alexa 488 fluorescence and red PI fluorescence were scored using 488 and 568 nm excitation light, respectively.

Preparation of cell lysate and western blot analysis

Murine 2237 fibrosarcoma cells were grown and treated with PVP as described earlier. After 24 or 48 hours of treatment, total and cytosolic protein lysates were prepared as described.[12] The protein content in the lysates was measured by *DC* Bio-Rad assay (Bio-Rad) as per the manufacturer's protocol. The proteins (50 μg) were resolved on 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (BioRad). Western blot analysis was done as described elsewhere.[12]

Mitochondrial membrane potential assay

The loss of mitochondrial membrane potential ($\Delta \Psi m$) was quantitatively determined by flow cytometry using the lipophilic cationic probe JC-1 dye (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide) (AnaSpec, San Jose, CA, USA). Briefly, after the PVP treatment for 48 hours, 1×10^6 cells were incubated in 10 μg JC-1 dye per ml PBS (pH 7.4) for 15 minutes at 37 °C in dark. Stained cells were washed, resuspended in 500 μl PBS (pH 7.4) and used for immediate FACS analysis.

Assay for caspase-3 and caspase -9 activity

Caspase-3 and caspase-9 activities were measured in cell lysates by the capacity to cleave their substrates DEVD-pNA and Ac-LEHD-pNA, respectively after treating 2237 cells with the PVP for 48 hours. Briefly, 2×10^6/ml cultured cells were incubated in lysis buffer (50 mM HEPES, 0.1% CHAPS, 1 mM DTT, 0.1 mM EDTA, pH = 7.4) for 5 minutes at 4 °C. The cytosolic extracts were collected by centrifugation and the protein concentration was determined using the *DC* protein assay. Aliquots of 20 μg of the samples were then incubated in the presence of caspase-3 and -9 substrates (Biomol, Plymouth Meeting, PA, USA). After 2 hours, absorbances were recorded at 450 nm in a Perkin Elmer Lambda II spectrophotometer.

Statistical analysis

Statistical analysis was performed using one-tailed Student's *t*-test assuming equal variances and statistical significance is expressed as $^*P < 0.05$, $^{**}P < 0.01$, $^{¶}P < 0.001$. All statistical analyses were conducted using the Microsoft Excel software (Washington, DC, USA).

Results

Purification and chemical identification of PVP

PVP was purified from a bacterium Ant5-2, which has been identified as a previously undescribed species by the 16S rRNA gene sequence and a panel of biochemical analyses (data not shown). The liquid chromatography, mass spectrometry and ^{1}H NMR results of the PVP (3-[5-(3-hydroxyl-1H-indol-3yl)-2-R_1-1H-pyrrol-3-ylidene]-2-R_1-1H-indol) (Fig. 1a) from Antarctic *Janthinobacterium* sp. Ant5-2[13] showed large molecular ion *m/z* (m + 1) 344 with the retention time at 3.5 minutes as shown in Fig. 1b. The structure of PVP is

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comparable to previously reported violacein pigment from *C. violaceum* (3-[1,2-dihydro-5-(5-hydroxy-1 *H*-indol-3-yl)-2-oxo-3*H*-pyrrol-3-ylidene]-1,3-dihydro-2*H*-indol-2-one).[7] In the ^{1}H NMR, four D_2O exchangeable protons were observed. Signals at δ 11.93, 10.76, and 10.65 were due to the NH signals and the signal at δ 9.36 can be assigned to the phenolic OH. Other aromatic proton signals appeared at identical shifts that were reported.[7]

PVP inhibits growth of murine 2237 and B16F10 cells but not of normal fibroblasts and keratinocytes

The treatment of 2237 fibrosarcoma cells with the PVP resulted in a significant reduction in the cell viability from 13% to 79% ($^{*}P < 0.05$ to $^{**}P < 0.01$) in a time and dose-dependent manner (Fig. 2a). To further check whether cytotoxicity of PVP was confined to only one skin cancer cell line or more, B16F10 cells were treated with PVP. This also resulted in significant reduction in the cell viability from 4% to 78% ($^{*}P < 0.05$) in a time and dose-dependent manner (Fig. 2b). To assess the cytotoxicity of PVP on the normal cells we used murine NIH3T3 fibroblast cell line. Since keratinocytes comprise more than 95% of skin epidermis and are responsible for the biochemical and physical integrity of skin,[14] we also treated murine keratinocytes C50 under identical conditions. Treatment of the NIH3T3 and C50 keratinocytes with up to 1 μM concentration of PVP exhibited more than 90% viable cells (Fig. 2c,d), whereas only 21% of the murine fibrosarcoma 2237 cells remained viable at 1 μM of PVP. In addition, Table 1 compares the results from this study with that of previous studies. It is apparent that PVP from *Janthinobacterium* Ant5-2 is more effective at lower concentrations on 2237 fibrosarcoma cells than violacein from *Chromobacterium violaceum* on leukemia HL60 or the colon cancer HCT116 cell lines.

PVP causes G0/G1 and G2/M phase cell cycle arrest in 2237 fibrosarcoma cells

Since cell cycle arrest is one of the upstream events leading to apoptosis, the cell cycle distribution was recorded after 24 hours of PVP treatment. As shown in Fig. 3a,b PVP treatment induced a G0/G1 arrest as well as a strong G2/M phase arrest concomitant with growth inhibitory effects. The distribution of cells in G0/G1 phase was 46.52%, 47.63%, 50.43%, and 52.77% at 0.1, 0.2, 0.5, and 1 μM concentrations of PVP, respectively. The distribution of cells in G2/M phase was 7.03%, 9.11%, 10.16%, and 23.32% at 0.1, 0.2, 0.5, and 1 μM concentrations of PVP, respectively. Further, there was decrease in cell number during S-phase (48.44–23.91%), which strongly implies that PVP results in the cell cycle arrest and inhibits the proliferation of murine 2237 fibrosarcoma cells.

PVP induces apoptosis in 2237 cells

Staining of 2237 fibrosarcoma cells with Annexin-V Alexa fluor 488 and Propidium iodide (PI) following treatment with various concentrations of PVP exhibited a significant increase in apoptosis when compared with controls (untreated 2237 cells) (Fig. 4a). Annexin-V specifically binds to phosphatidylserine and has been employed as a useful tool for detecting apoptotic cells. Apoptotic cells (green fluorescence) were found to be increased in PVP treated cells in a dose-dependent manner (Fig. 4a). Further, Annexin V-positive (apoptotic cells) populations were analyzed by flow cytometry (Fig. 4b). PVP treatment induced apoptosis and resulted in cell death from 26.8% to 78% at increasing concentration (0.1–1 μM) (Fig. 4c).

PVP modulates the expression of Bcl-2 family proteins in 2237 cells

The immunoblot analysis results showed that the PVP treatment of murine 2237 fibrosarcoma cells increased the expression of Bax with concomitant decrease in the expression of Bcl-2 in a PVP dose-dependent manner (Fig. 5a). Thus, PVP treatment resulted in the alteration in Bax/Bcl-2 ratio in favor of apoptosis (Fig. 5a).

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PVP enhances the release of cytochrome c and induces loss of mitochondrial membrane potential in murine 2237 cells

Loss of mitochondrial membrane potential in the cells triggers the release of cytochrome c from the mitochondria to the cytosol,[15] which in turn contributes to the activation of caspases and subsequent apoptotic cell death. On disruption of the mitochondrial membrane potential, the fluorescence emission of JC-1 dye changes from red (multimer J-aggregates emitting fluorescence light at 590 nm) to green (monomeric form emits light at 527 nm) after excitation at 490 nm. As shown in Fig. 5b,c, the number of cells with green fluorescence increased from 0.8% in control untreated cells to 5.1%, 6.7%, 26%, and 62.6% in PVP treated cells. Western blot analysis of the cytosolic fractions of the cellular lysates revealed that PVP caused a dose-dependent increase in the release of cytochrome c to the cytoplasm (Fig. 5d), thus confirming the role of PVP in the disruption of mitochondrial membrane potential.

PVP induces activation of caspases and cleavage of PARP protein

Immunoblot analysis showed decreased expression of procaspase-3 and increased expression of cleaved caspase-9 and -3 after 48 hours of PVP treatment (Fig. 6a). PARP is a 116 kDa protein that is cleaved into 85 and 30 kDa fragment during apoptotic cell death. Thus, the cleavage of PARP is regarded as hallmark for the induction of apoptotic response. There was significant increase in the amount of 85 kDa fragment in the 0.5 and 1 μM PVP-treated cells as compared to untreated control cells (Fig. 6a). The PVP-induced activation of caspase-9 and caspase-3 in 2237 fibrosarcoma cells were further confirmed using colorimetric caspase-9 and caspase-3 activity assays. Treatment of 2237 cells with PVP for 48 hours resulted in a significant (** $P < 0.01$, ¶ $P < 0.001$) increase in both caspase-3 and caspase-9 activity in a dose-dependent manner as compared to untreated control cells (Fig. 6b,c), thus confirming the involvement of caspase-9 and -3 activation in apoptotic cell death of 2237 cells.

Discussion

The development of mechanism-based prevention and treatment of cancer by the natural compounds capable of inducing apoptosis is now gaining the attention of scientists and pharmaceutical companies.[16] Because of its cytotoxic effect at low concentrations on both the fibrosarcoma and melanoma cells and non toxicity to normal fibroblasts and keratinocytes, PVP can potentially be used as an effective chemotherapeutic agent against skin cancer, especially fibrosarcoma. Therefore, the results from this study provide an insight into the molecular mechanism that causes cell death in fibrosarcoma following treatment with PVP.

In order to control and arrest tumor growth, the control of cell cycle progression is an efficient strategy.[17] Our *in vitro* data demonstrated that the treatment of 2237 fibrosarcoma cells with PVP not only induced G0/G1 phase arrest but the cells that escaped G0/G1 phase were stopped at the next check point G2/M phase with obvious decrease of cells in the S-phase. Our data also demonstrated decreased expression of cyclin and cyclin-dependent kinases (cyclin D1, Cdk2, Cdk4, and Cdk6) and increased expression of cyclin-dependent kinase inhibitors (Cip1/p21 and Kip1/p27), which forms heterotrimeric complexes with the G_1/S Cdks and cyclins and inhibit their activity. Further, PVP decreased Cdc2 and cyclin B1, whose activation is required for transition from G2 to M phase of the cell cycle.[17] It has been reported that the p53 and p21 are necessary to maintain a G2 arrest following DNA damage, because tumor cells lacking these proteins enter into mitosis with accelerated kinetics.[18,19] In this study, we observed that treatment of 2237 fibrosarcoma cells with PVP resulted in the increased level of p53 and p21 protein (Fig. 3c). These results suggested that

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the p53-dependent reduced expression of cyclin B1 and Cdc2 may also be involved in PVP-induced G2/M phase arrest, leading to cell growth inhibition and possible apoptotic death. In one of the studies, it has been shown that a small molecular weight compound CP-31398 was able to restore wild-type functionality to mutant p53, thereby reducing the risk of developing sunlight-induced human skin cancers carrying mutant p53.[20]The 2237 fibrosarcoma cells have a p53 mutation[21] and our results showed an increased expression of p21 and Bax proteins with an increased expression of p53. Therefore, it seems plausible that PVP might be restoring the activity of mutant p53 resulting in the cell cycle arrest and release of cytochrome c resulting in apoptosis.

Consistent with earlier reports, in this study, we found that treatment of 2237 fibrosarcoma cells with PVP resulted in a dose-dependent decrease in the levels of anti-apoptotic protein (Bcl-2) and simultaneous increase in proapoptotic protein Bax (Fig. 5a), which forms the major apoptotic signal transduction cascade associated with programmed cell death.[22] This alteration may be responsible for the disruption of mitochondrial membrane potential and increased release of cytochrome *c* to cytosol (Fig. 5c,d). Further, this effect of PVP led to the dose-dependent activation or cleavage of caspases-9, -3 and PARP. In conclusion, our study indicates that PVP from Antarctic *Janthinobacterium* sp. Ant5-2 inhibits growth, induces G0/G1 and G2/M cell cycle arrest and apoptotic cell death of murine 2237 fibrosarcoma cells via mitochondrial pathway. Also, we have shown that PVP has antiproliferative effect on melanoma B16F10 cells. We believe that future investigation of the targets for PVP in preventing skin cancer and testing its effectiveness *in vivo* would help determine its potential as a chemotherapeutic agent.

Acknowledgments

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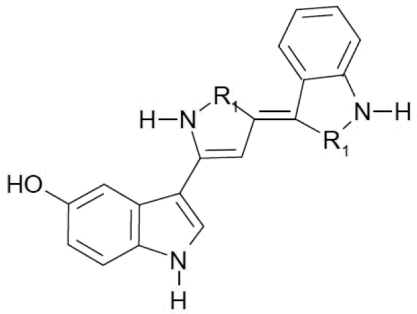
A



R_1 = undetermined

B

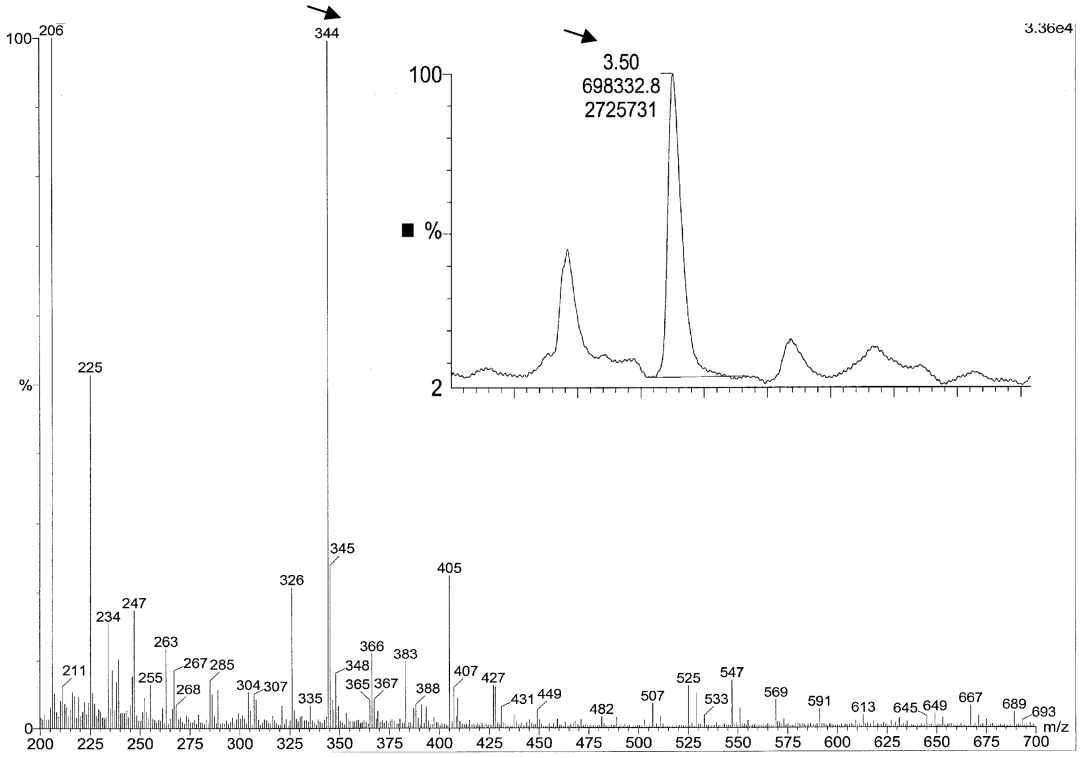


Figure 1.

(a) The deduced molecular structure of PVP (3-[5-(3-hydroxyl-1H-indol-3yl)-2-R_1-1H-pyrrol-3-ylidene]-2-R_1-1H-indol) from mass spectra and proton (^{1}H) NMR (Bruker ARX 700 spectrometer). (b) LC-MS chromatogram showing the peak (ion selected at *m/z* 344) of PVP with the retention time at 3.5 minutes as indicated by short arrows.

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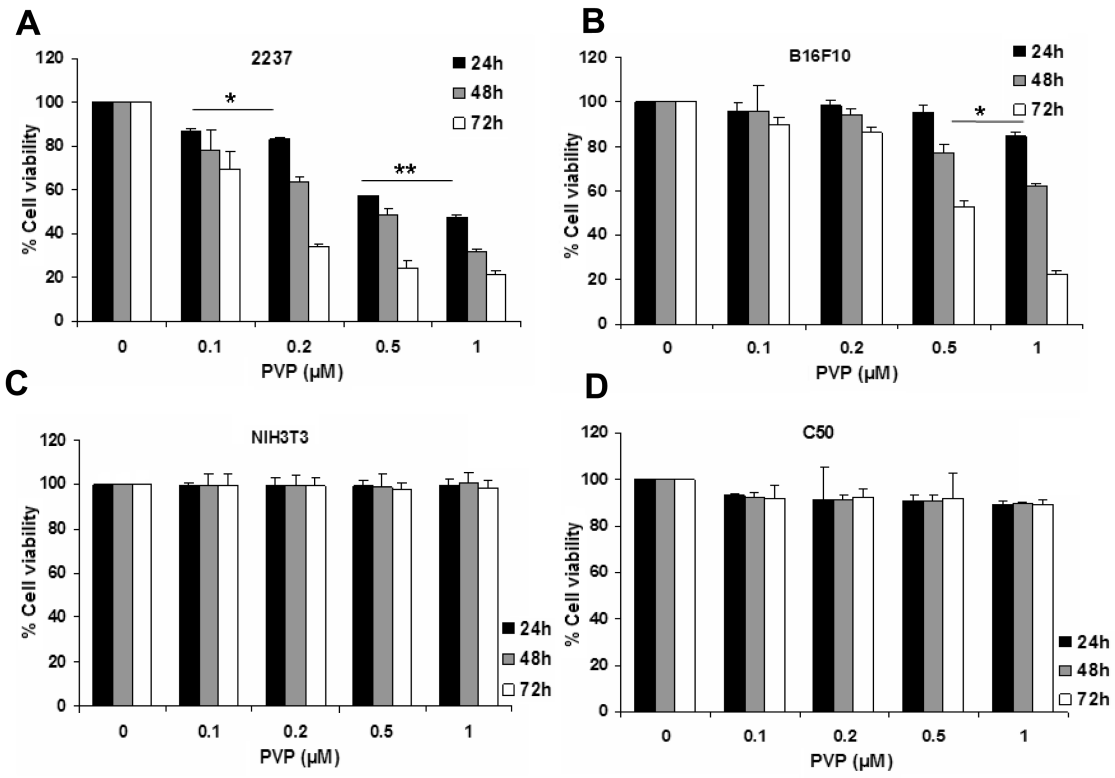

Figure 2.

Dose- and time-dependent effect of PVP on the proliferation of murine (a) 2237 fibrosarcoma cells (b) B16F10 melanoma cells (c) NIH3T3 fibroblasts and (d) C50 keratinocytes was determined using MTT assay. The values are represented as the percentage cell viability where DMSO treated cells (0.02% DMSO) was regarded as 100%. The data represents the mean ± SD of three independent experiments each conducted in quadruplicate (*$P < 0.05$, **$P < 0.01$)

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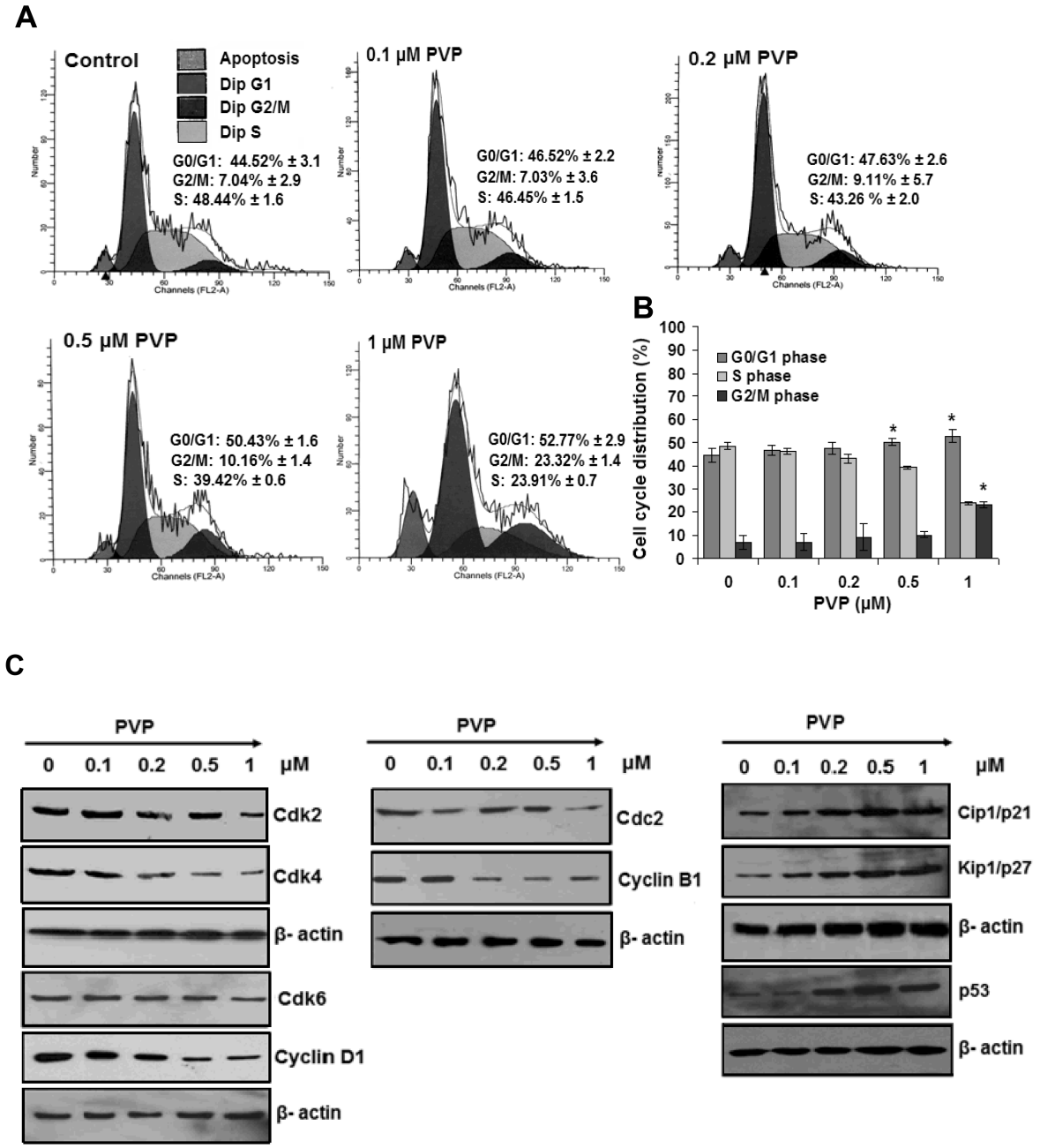

Figure 3.

(a) The representative cellular DNA histograms with apoptosis peak showing the percentages of cell-cycle phases ± SD from three independent experiments. (b) Graphic representation of the percentages of cell cycle phases ± SD (*$P < 0.05$). (c) Differential expression of cell cycle regulatory proteins Cdk2, Cdk4, Cdk6, Cyclin D1, Cyclin B1, Cdc2, p21, p27 and p53 in 2237 cells after treatment with PVP. Equal loading of protein was confirmed by stripping the immunoblot and reprobing it for β-actin.

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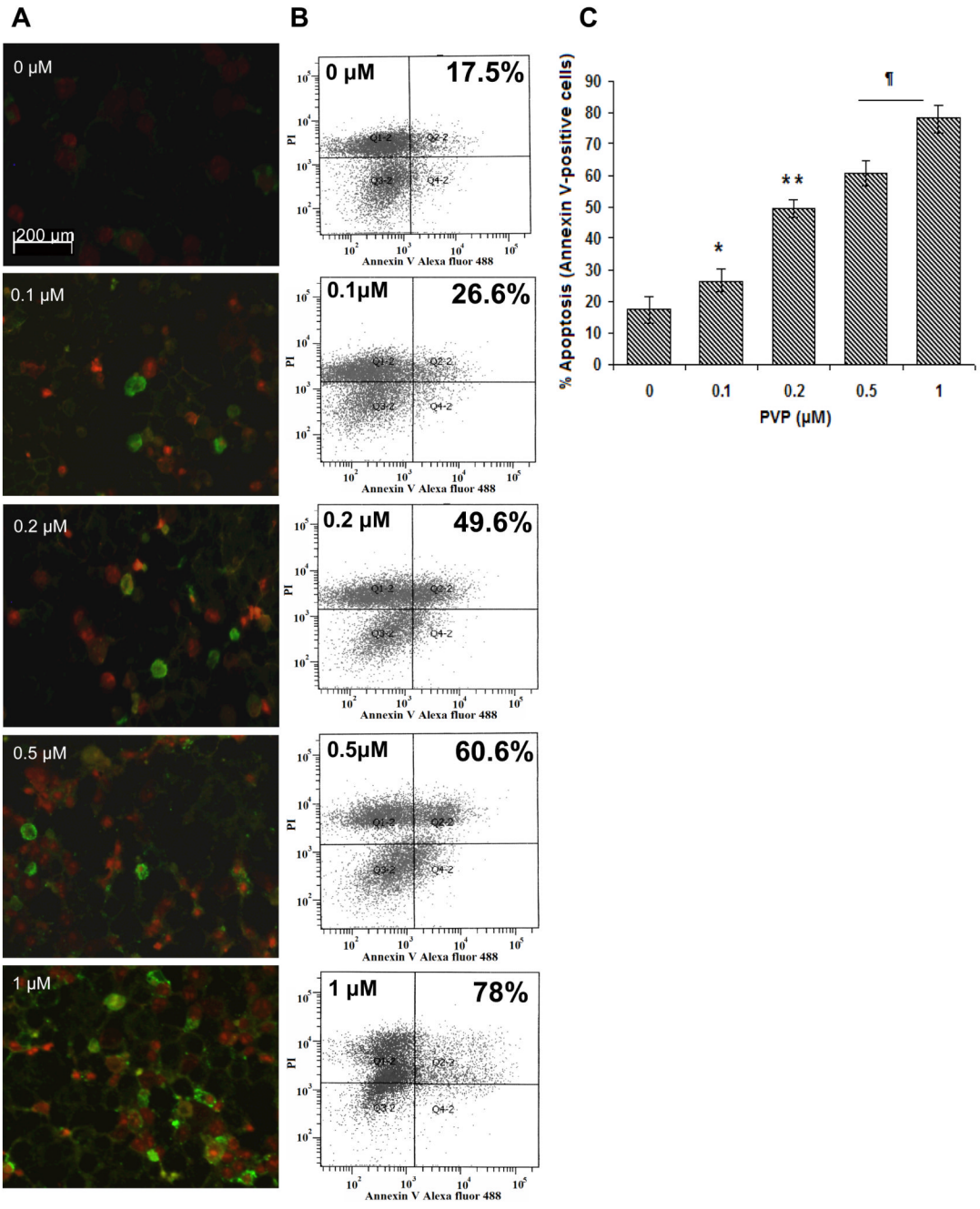

Figure 4.

(a) After 48 hours of treatment 2237 cells were stained using Annexin V-Alexa Fluor488 Apoptosis Vybrant Assay Kit following manufacturer's protocol for fluorescence microscopy and the green fluorescent Annexin-V positive cells were observed under fluorescence microscope (20X). (b) The FACS histograms of the Annexin V-positive populations of 2237 cells after 48 hours of treatment with PVP at different concentrations. The mean percentages of apoptotic cells (Annexin-V positive cells) are shown in the panels above. (c) The mean percentages of apoptotic cells (Annexin-V positive cells) ± SD from two independent experiments are represented by the graph (*$P < 0.05$, **$P < 0.01$, ¶$P <$ 0.001).

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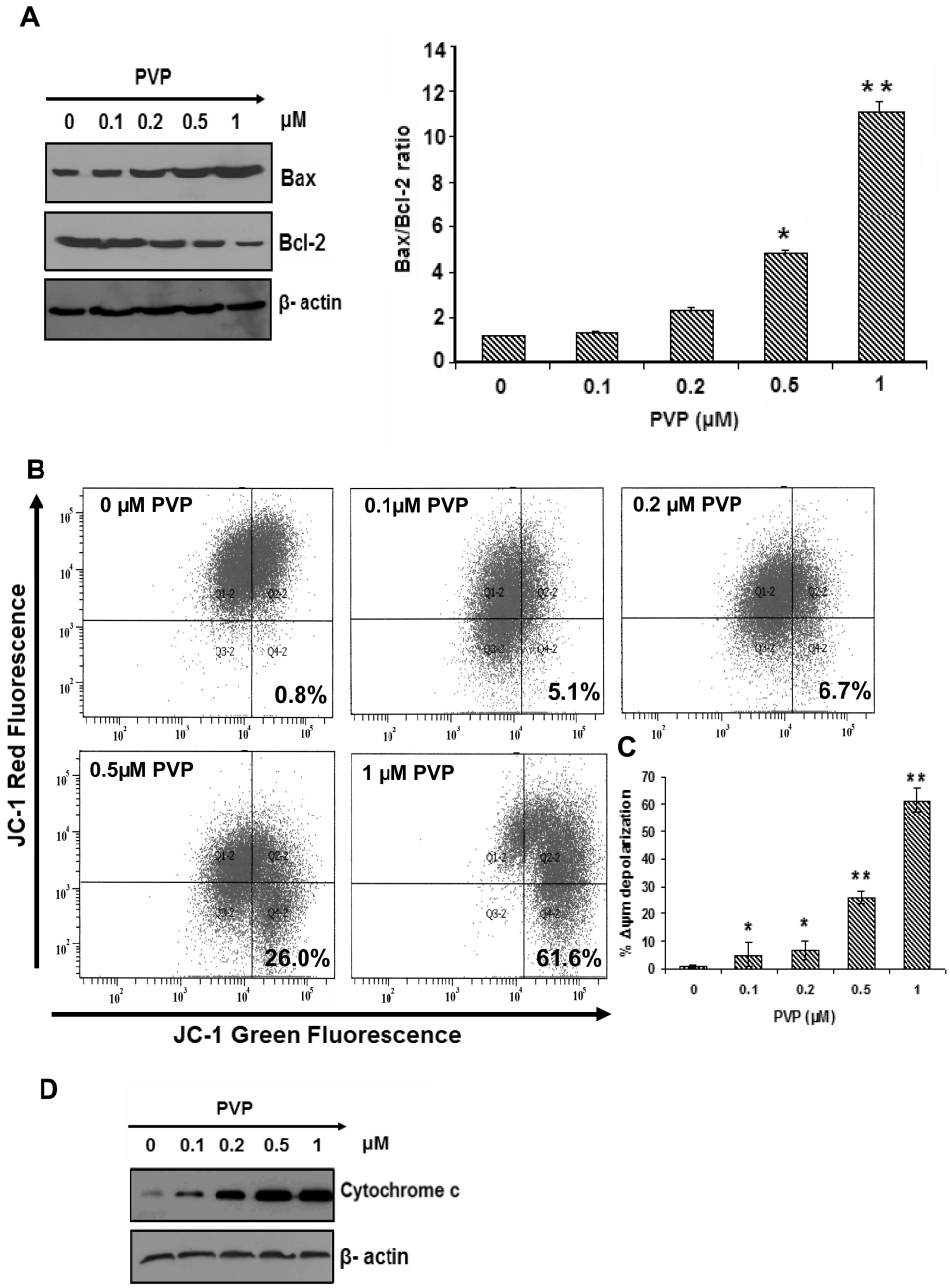

Figure 5.

(a) The Western blot data for the expression of the Bax and Bcl-2 proteins are presented in the bar graphs as percentage of Bax/Bcl-2 ratio ± SD after treatment of 2237 cells with PVP (0–1 μM) for 48 hours (*$P < 0.05$, **$P < 0.01$). (b) Dose-dependent loss of the mitochondrial membrane potential after treatment of 2237 cells with PVP (0–1 μM) for 48 hours and JC-1 staining. The representative dot plots from a single analysis are shown with the percentage of the cells in the lower right (LR) quadrant that emit only green fluorescence indicating the depolarized mitochondrial membrane ($\Delta \Psi$m). (c) Graphic representation of mean values of the percentage of cells with collapsed $\Delta \Psi$m ± SD from two experiments. (d)

Cytosolic fractions were prepared from the same treatment groups and subjected to Western blot analysis to detect the levels of cytochrome c.

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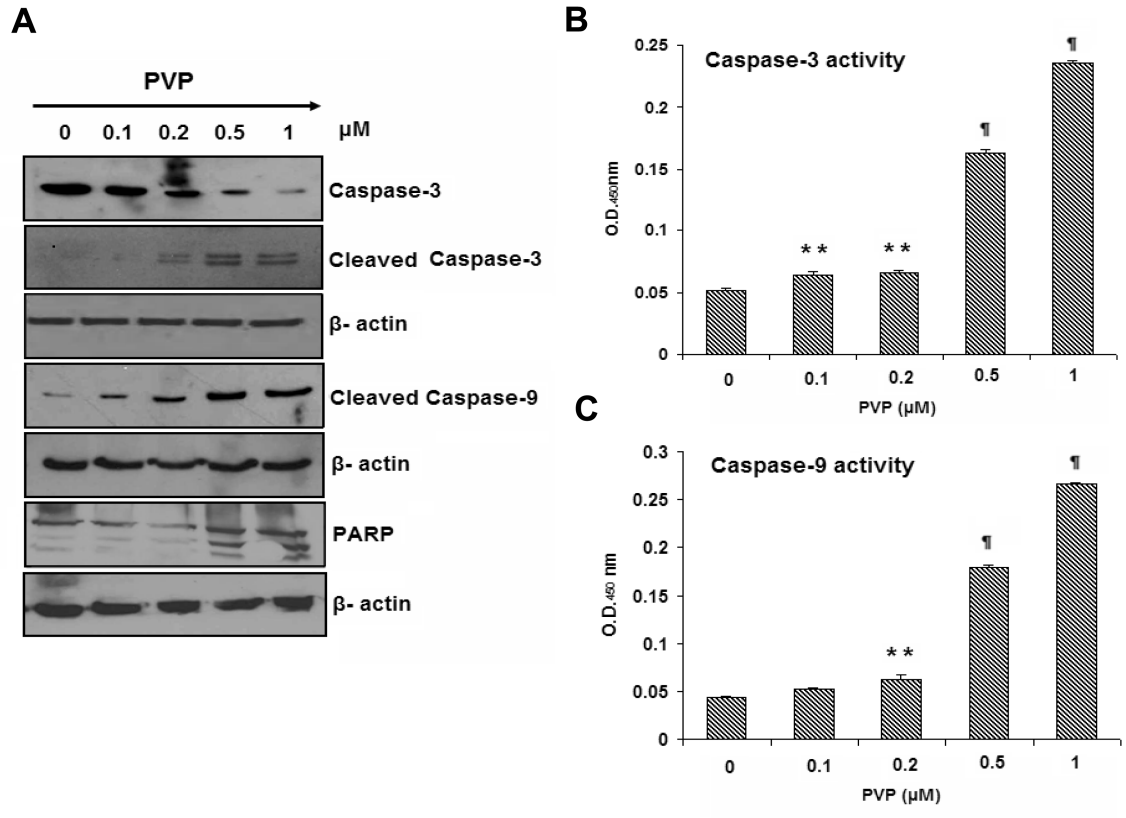

Figure 6.

(a) Western blot analysis was performed to detect the levels of caspase-3, cleaved caspase-9 and -3, and PARP. Equal protein loading was checked by probing stripped blots for β-actin, and a representative blot is shown from two independent experiments with identical observations. (b) The caspase-3 activity was measured in cell lysate samples obtained from the treatment groups using the substrate DEVD-pNA in a colorimetric assay and (c) similarly, the caspase-9 activity was measured using the substrate Ac-LEHD-pNA. Data are representative sets from two independent experiments expressed as mean absorbance at 450 nm ± SD (** $P < 0.01$, ¶ $P < 0.001$)

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Table I

Comparison of effectiveness of violacein and PVP on different cell lines

Cell lines	% Cell death			Effective concentration of violacein/PVP (μM)	IC50 (50% cell death) (μM)	Reference
	24 h	48 h	72h			
Skin cancer (2237)	87%	93%	93%	1 (PVP)	0.5 (PVP)	This study, 2011
Leukemia (HL60)	65%	-	-	2 (violacein)	0.7 (violacein)	Ferreira et al, 2004
Colon Cancer (HCT116)	-	93%	-	3 (violacein)	~1-2 (violacein)	Kodach et al, 2006

Note: Normal keratinocytes (C50) and normal fibroblasts (NIH3T3) showed ≤10 % cell death upon PVP treatment for 24, 48 and 72 h.